


# Hepatic transcriptome profiling indicates differential mRNA expression of apoptosis and immune related genes in eelpout (Zoarces viviparus) caught at Göteborg harbor, Sweden

Aquatic Toxicology 130–131 (2013) 58–67

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

# Hepatic transcriptome profiling indicates differential mRNA expression of apoptosis and immune related genes in eelpout (*Zoarces viviparus*) caught at Göteborg harbor, Sweden

Noomi Asker[a,*], Erik Kristiansson[b], Eva Albertsson[a], D.G. Joakim Larsson[c], Lars Förlin[a]

[a] Department of Biological and Environmental Sciences, University of Gothenburg, Gothenburg, Sweden
[b] Department of Mathematical Statistics, Chalmers University of Technology, Gothenburg, Sweden
[c] Department of Infectious Diseases, Institute of Biomedicine, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

ARTICLE INFO

*Article history:*
Received 5 June 2012
Received in revised form
12 December 2012
Accepted 18 December 2012

*Keywords:*
Transcriptomics
Chronic exposure
Pollution
Eelpout
Microarray

ABSTRACT

The physiology and reproductive performance of eelpout (*Zoarces viviparus*) have been monitored along the Swedish coast for more than three decades. In this study, transcriptomic profiling was applied for the first time as an exploratory tool to search for new potential candidate biomarkers and to investigate possible stress responses in fish collected from a chronically polluted area. An oligonucleotide microarray with more than 15,000 sequences was used to assess differentially expressed hepatic mRNA levels in female eelpout collected from the contaminated area at Göteborg harbor compared to fish from a national reference site, Fjällbacka. Genes involved in apoptosis and DNA damage (e.g., SMAC/diablo homolog and DDIT4/DNA-damage-inducible protein transcript 4) had higher mRNA expression levels in eelpout from the harbor compared to the reference site, whereas mRNA expression of genes involved in the innate immune system (e.g., complement components and hepcidin) and protein transport/folding (e.g., signal recognition particle and protein disulfide-isomerase) were expressed at lower levels. Gene Ontology enrichment analysis revealed that genes involved biological processes associated with protein folding, immune responses and complement activation were differentially expressed in the harbor eelpout compared to the reference site. The differential mRNA expression of selected genes involved in apoptosis/DNA damage and in the innate immune system was verified by quantitative PCR, using the same fish in addition to eelpout captured four years later. Thus, our approach has identified new potential biomarkers of pollutant exposure and has generated hypotheses on disturbed physiological processes in eelpout. Despite a higher mRNA expression of genes related to apoptosis (e.g., diablo homolog) in eelpout captured in the harbor there were no significant differences in the number of TUNEL-positive apoptotic cells between sites. The mRNA level of genes involved in apoptosis/DNA damage and the status of the innate immune system in fish species captured in polluted environments should be studied in more detail to lay the groundwork for future biomonitoring studies.



## 1. Introduction

The aquatic environment is often the ultimate sink for many of the pollutants released from sources such as industry, agriculture and households. To study the exposure and effects of environmental pollutants, biomarkers are often used. Many of the well characterized biomarkers are not always differentially expressed between fish captured in clean and polluted areas. This may be due to compensatory processes that occur during chronic exposures, not observed in acute exposure scenarios. It may also be due to interactions between multiple chemicals or due to the influence of other abiotic and biotic factors that are difficult to control in field studies. Furthermore, the chemical complexity of many polluted areas makes it challenging to make a priori prediction about what responses to expect and how to subsequently study them in exposed animals. Hence, it is important to identify and evaluate novel biomarkers for use in future field studies in polluted environments.

Gene expression DNA microarray is a high-throughput technique, in which the mRNA expression levels of tens of thousands of genes are typically measured simultaneously. Such "global"

*Abbreviations:* BEEP, Biological effects of environmental pollution in marine coastal ecosystems; HELCOM, The Baltic marine environment protection commission, also known as the Helsinki Commission; OSPAR, Convention for the protection of the marine environment of the North-East Atlantic.

* Corresponding author at: Department of Biological and Environmental Sciences, University of Gothenburg, Box 463, SE-405 30 Gothenburg, Sweden.
Tel.: +46 31 7863685; fax: +46 31 416729.
*E-mail address:* noomi.asker@bioenv.gu.se (N. Asker).

0166-445X/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquatox.2012.12.017

transcriptome analyses constitute an exploratory tool, in the sense that the vast amount of data retrieved can be used to generate hypotheses about molecular changes associated with, for example, different types of stress or disease. Microarray analysis can therefore be used in aquatic ecotoxicology to (i) generate hypotheses on the mode of action of toxic compounds/mixtures, (ii) identify early warning signs of toxic exposure, (iii) increase knowledge about affected biological processes and pathways, (iv) provide insight into the overall health status of organisms living in polluted areas and (v) identify responses to exposure that are otherwise difficult to predict based on traditional biomonitoring studies using classical biomarkers. Combining ecotoxicology with DNA microarray technology into ecotoxicogenomics is a powerful tool in the environmental risk assessment process (Fent and Sumpter, 2011; Van Aggelen et al., 2010). However, to date only a few ecotoxicogenomics studies have been performed using large-scale genome-wide transcriptome analysis on field-sampled fish populations (Baker et al., 2009; Falciani et al., 2008; Fisher and Oleksiak, 2007; Lie et al., 2009; Moran et al., 2007; Oleksiak, 2008; Roling et al., 2007; Williams et al., 2011).

In this study, the eelpout (*Zoarces viviparus*) was used as bioindicator of contaminant exposure. The eelpout is a bottom-dwelling fish species found in the coastal areas of Northern Europe. As the eelpout is relatively stationary, responses detected in the fish will likely be a true reflection of the environmental conditions, including chemical levels, within the area where the fish is captured (Jacobsson et al., 1986). The eelpout is viviparous and has been recommended as sentinel species to monitor the effects of pollutants and the status of the marine environment by national environmental protection agencies, international commissions (HELCOM 2008 and OSPAR 2006) and the International Council for Exploration of the Sea (ICES 2007). In Sweden, the eelpout has been part of the national biomonitoring program for more than 25 years and it has also been used in other research projects specifically assessing the health status of the eelpout (Carney Almroth et al., 2005; Larsson and Förlin, 2002; Ronisz et al., 1999; Sturve et al., 2005). The eelpout has also been utilized in monitoring and research programs run in Denmark (Strand, 2007; Strand et al., 2004, 2009) and Germany (Gercken et al., 2006; Gercken and Sordyl, 2002) and it was also used as sentinel species when validating biomarkers of contaminant exposure in the EU founded BEEP project (Lehtonen et al., 2006). For a recent review of the use of the eelpout in marine environmental monitoring, see Hedman et al. (2011).

Göteborg harbor is Scandinavia's largest port located at the outlet of the river Göta Älv on the Swedish west coast. The main cargo transported into the harbor is oil with a volume of more than 20 million tons every year. The harbor water is polluted with various anthropogenic substances, and chemical analyses of the sediment indicate contamination with several toxic compounds, including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), tributyltin, dioxins and heavy metals such as Hg (Brack, 2002; Brack and Stevens, 2001; Magnusson et al., 1996). The eelpout has been used as sentinel species in the harbor not only during the yearly assessment of the chronic effects of environmental pollutants but also during studies of more acute environmental changes, such as following harbor dredging or an oil spill that released more than ten tons of bunker oil (Carney Almroth et al., 2005; Sturve et al., 2005).

We have previously sequenced the eelpout liver transcriptome using massively parallel pyrosequencing (Kristiansson et al., 2009). The sequence data were used to assemble genes and to develop an eelpout oligonucleotide microarray. The array contains several categories of genes of particular interest for ecotoxicological research, including different cytochrome P450 variants, heat shock proteins and genes related to oxidative stress. In addition, several commonly used biomarkers, such as vitellogenin, zona pellucida proteins and metallothionein, are represented on the array. In the present study, we used microarrays and quantitative PCR to analyze the hepatic transcriptome of eelpout captured at Göteborg harbor and at a well-characterized reference site north of Göteborg harbor. Attempts were also made to link some of the differential mRNA expressions to phenotype. The major aims were to search for novel potential biomarkers for future biomonitoring studies and to investigate possible signs of stress responses in fish collected from a chronically polluted area.

## 2. Materials and methods

### 2.1. Sampling of wild fish

Eelpout, *Z. viviparus*, were caught using fyke nets operated by local fishermen in November of 2006 and 2010 from two different locations on the Swedish west coast: Göteborg harbor (lat. 57.6866, long. 11.752), located in the outlet of the Göta älv river and Fjällbacka (lat. 58.6440, long. 11.2457), which is a national reference site located 150 km north of Gothenburg. The fish lengths ranged from 20.8 to 34.0 cm, and all fish collected were sexually mature. Fish sampling and dissection of livers were performed as described earlier (Sturve et al., 2005). In total, 9–10 eelpout females were individually analyzed per site, per year. The females eelpout used in this study were also part of the annual biomonitoring of the Swedish west coast, performed in collaboration with the Swedish environmental protection agency (Förlin, 2008). All animal experiments were approved by the local animal committee in Gothenburg, Sweden.

### 2.2. Total RNA isolation

Liver samples were individually homogenized in lysis buffer (RNeasy mini plus kit (Qiagen, Hilden, Germany)) using a TissueLyzer (Qiagen) at 25 Hz for $2 \times 4$ min (each tube contained a steel bead, Ø5 mm (Qiagen)). Total RNA isolation was automated using a QIAcube (Qiagen) and an RNeasy mini plus kit according to the manufacturer's instructions (using 50% EtOH). Genomic DNA was removed via the gDNA Eliminator spin columns provided with the RNeasy mini plus kit. RNA quality and quantity were assessed using an Experion automated electrophoresis (Bio-Rad, Hercules, CA, USA) and a Nanodrop ND1000 (NanoDrop Technologies, Wilmington, DE, USA), respectively.

### 2.3. Microarray design

A custom-made eelpout oligonucleotide microarray was designed for the Geniom platform (Febit, Heidelberg, Germany). Probes were generated as described earlier (Kristiansson et al., 2009) using OligoArray 2.1 with a probe length of 50 nucleotides. The probes generated exceeded the maximum amount of probes for each array, and therefore, a selection was needed. Eelpout transcript homologues for genes from the Comparative Toxicogenomics Database (CTD) and the Pharmacogenomics Knowledge database (PharmGKB) were added to the microarray as well as eelpout homologs for all cytochrome P450 (CYP) genes annotated in *Danio rerio*. We also included genes found to be differentially expressed in previous fish exposure studies and/or genes relevant to ecotoxicology selected from the literature. The prediction of eelpout homologs was performed using the protein database UniProt, in addition to five available fish genomes and the human genome, as previously described (Kristiansson et al., 2009). The sequence comparison was performed using the Washington University Basic Local Alignment Search Tool 2.2.6 (http://blast.wustl.edu) in tbastx mode, with an e-value cut-off of $10^{-10}$. The microarrays were finally synthesized in situ on each of the eight separate subarrays (15,230

probes/subarray) on the microarray biochip using the 5′ standard synthesis set for Geniom.

### 2.4. Microarray hybridization

A total of 19 microarray hybridizations were performed on eelpout females captured in 2006, using ten individuals from Fjällbacka and nine individuals from Göteborg harbor. Biotinylated complementary RNA (cRNA) was prepared using the Message Amp II-Biotin Enhanced single round Amplification kit (Ambion® Inc., Austin, TX, USA), starting with 1 μg of total RNA and performing the in vitro transcription for 14 h. Following the in vitro transcription, the cDNA was used as a template for the linear amplification of biotinylated cRNA. Chemical fragmentation of the cRNA was performed at 94 °C for 35 min, using 40 mM Tris-acetate, 100 mM K-acetate and 30 mM Mg-acetate at a concentration of 1.6 μg cRNA $\mu l^{-1}$. Fragmented cRNA (6 μg $array^{-1}$) was dissolved in hybridization buffer, (final concentration: 5× SSPE (saline–sodium phosphate–EDTA buffer), 20% formamide, 0.5 mg BSA/ml, 0.1× TE (Tris–EDTA buffer) and 0.1 mg mouse CotI (Invitrogen Carlsbad, CA, USA), 0.01% Tween 20. Samples were denatured at 95 °C for 3 min and hybridized to the arrays at 45 °C for 15 h. The arrays were washed using 6× SSPE at 25 °C and 0.5× SSPE at 45 °C. Fluorescent staining of the hybridized cRNA was performed by incubating the arrays in SAPE (streptavidin, R-phycoerythrin conjugate, Invitrogen) diluted in 6× SSPE (5 μg SAPE $ml^{-1}$). Signal amplification was performed using biotinylated α-streptavidin antibodies (Vector Laboratories, Burlingame, CA, USA), followed by an additional incubation with SAPE, according to the Consecutive Signal Enhancement protocol (Febit, Heidelberg, Germany). Hybridization, washing, signals amplification and detection were performed using the automated microarray platform, Geniom® Analyzer (Febit, Heidelberg, Germany).

### 2.5. Statistical analysis on microarray transcriptome data

The microarray data were analyzed as described earlier (Cuklev et al., 2011) using the statistical language R 2.12.1 (www.r-project.org) and Bioconductor 2.5. Technical artifacts were removed using quantile–quantile normalization and the data were log2-transformed. Differential expression patterns between eelpout females from Göteborg harbor and from the reference site in Fjällbacka were identified by the moderated *t*-statistic. The Benjamini–Hochberg false discovery rate (FDR) was used to estimate the proportion of false positives. Only genes with an estimated FDR less than 0.2 (corresponding to a *p*-value of 0.043) were considered for further analysis, limiting the proportion of false positives to 20%. Functional enrichment analysis for Gene Ontology (GO; http://www.geneontology.org/) was carried out using the GOrilla web service against the human database. For the functional enrichment analysis, genes were selected based on a FRD < 0.2 and a two-fold in differential expression. All genes on the chip were used as the background reference set.

### 2.6. Quantitative PCR analysis

A total of 19 quantitative PCR (qPCR) analyses were performed on hepatic total RNA from the eelpout females captured in 2006 using the same individuals as those used for the microarray study (nine females from Göteborg harbor and ten females from Fjällbacka). A total of 20 qPCR analyses were also performed on hepatic total RNA from the eelpout females captured in 2010 (ten females from Göteborg harbor and ten females from Fjällbacka). Total RNA (1 μg) was reverse-transcribed in duplicate to cDNA, using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, USA) and following the manufacturer's protocol. Transcripts obtained from the previously published eelpout sequencing project (Kristiansson et al., 2009) were uploaded to Molecular beacon (Bio-Rad) where a primer pair was designed for each gene. The amplification reaction was performed using the fluorescent dye, iQ™ SYBR®Green Supermix (Bio-Rad) with 20 μg cDNA sample giving a final reaction volume of 20 μl/well. The qPCR was performed in an iQ5 Thermal cycler (Bio-Rad) on a 96-well qPCR plate. The concentration and annealing temperature for each primer pair were optimized to ensure the efficiency between 95 and 105%. The general protocol was as follows: denaturation at 95 °C for 20 s, annealing for 20 s and elongation at 72 °C for 30 s. The cycle was repeated 40 times and was followed by a melt curve to confirm specific amplification. Primer sequences, primer concentration and annealing temperature is presented in Table 1. To exclude primer and DNA contamination, pooled samples were used to check for no template controls (NTC) and no reverse transcriptase controls (NoRT). Statistical analyses were performed using IBM SPSS statistics 19 (IBM Corporation, NY, USA). Levene's test for equality of variances was used to assure that the data fulfilled the homogeneity criteria required to perform a two-tailed *t*-test. When the criteria were not met, a Bonferroni correction was performed. The significance level ($\alpha$) was set to 0.05. Ubiquitin was identified as a stable suitable reference gene (with a *p*-value of 0.69 for samples collected in 2006, and 0.82 in samples collected in 2010, using two-tailed *t*-tests). The qPCR data were analyzed using the cycle threshold (ct) values for each gene of interest and subtracting the ct value of the reference gene.

### 2.7. TUNEL assay

The amount of apoptotic hepatocytes were estimated using the TUNEL assay on eelpout females captured in 2006 (seven females form both Göteborg harbor and seven females from the reference site in Fjällbacka). Liver samples were fixed in paraformaldehyde and embedded in paraffin followed by sectioning. Sections were treated as set out in the ApopTag® Plus in situ apoptosis fluorescein detection kit (Merck Millipore, Merck KGaA, Darmstadt, Germany), staining fragmented DNA in apoptotic cells. Sectioning and labeling was performed by Histo-Center AB (Gothenburg, Sweden). Apoptotic hepatocytes in liver sections were quantitated by counting the number of cells with fluorescent nucleus in 30 random positions with a 40× objective (NA 0.85) estimating the numbers of TUNEL-positive cells per 1000 cells in each tissue section. A two-tailed *t*-test was used for statistical comparison.

## 3. Results

### 3.1. Transcriptome analysis

Hepatic transcriptome analysis was performed on eelpout females captured in 2006 at Göteborg harbor and at the reference site in Fjällbacka. According to the microarray analysis, 325 genes had significantly differential mRNA levels (FDR < 0.2 and at least a two-fold in differential expression). Of these, 211 were unique genes with known annotations, 53 of which had higher mRNA expression levels and 158 of which had lower mRNA expression levels in eelpout captured in Göteborg harbor compared to eelpout captured in the reference site in Fjällbacka. For a ranked list of the top genes (FDR < 0.2), see the supplementary data.

A heatmap was generated using the top 200 most highly differentially transcribed genes (based on FDR) to visualize the differences in the mRNA expression levels between individual samples. Despite the high individual variation, a clear separation was observed between the sites (Fig. 1). This finding was further

**Table 1**
Primes for quantitative PCR.

| Gene | Accession no. | Primer | | Primer (nM) | Annealing temp. (°C) |
|---|---|---|---|---|---|
| | | FW 5′–3′ | RE 5′–3′ | | |
| CYP24 | ZOVI0038000 | GAG GTG TTG GTG GAC TTC GTT G | TCG TAG AGG ATG AGG CAG ATG G | 300 | 62.0 |
| PDI | ZOVI0025258 | TAT CCA GGT TGA CAT CGG | GTT CCA GCA TCC TTA GTG | 300 | 56.8 |
| SRP09 | ZOVI0036724 | CTT ACT TTC AGA CTT GGG AGG AG | CAG AGA TTA CCG TCG CAG TG | 300 | 59.0 |
| HSP70A5 | ZOVI0038668 | TAT CCA GGT TGA CAT CGG | GTT CCA GCA TCC TTA GTG | 300 | 53.5 |
| DDIT4 | ZOVI0007782 | CTG GTG CTG CGG CTA GAT TC | ACG ACT GGA GTG GAA GGA GAC | 500 | 60.4 |
| Diablo | ZOVI0033152 | TGT TGG TCA GTG TTC AGT GTG | TGG TCC GTA GCA GTG ATA GG | 300 | 58.8 |
| Heps | ZOVI0038001 | CTC TGC TGT CCC AAT CAC | GCT TCT CTC TGT TGT TAT ACG | 700 | 55.9 |
| C7 | ZOVI0005671 | TAC GAC TAC TCT GCC TAC C | TCA CCT TCC TCC AAC ACC | 300 | 55.3 |
| LysC | ZOVI0046979 | GGA GTC GGA TTA CAA CA | CAT CAT CAG TCA GAA GGT C | 700 | 56.9 |
| Ubiq | ZOVI0023393 | GCT TGA GGA TGG ACG CAC AC | CCA CGC AGA CGC AGA ACC | 500 | 58.3 |

Forward (FW) and reverse (RE) primers used for quantitative PCR with respective gene name, ZOVI (*Zoarces viviparus*) accession numbers, primer concentration and annealing temperature.

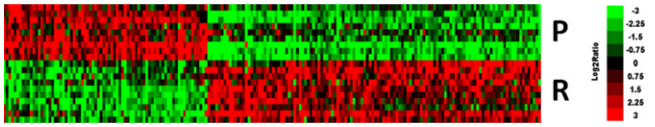


**Fig. 1.** Heatmap on hepatic transcriptome data from individual eelpout female captured in the polluted (P) waters of Göteborg harbor (nine samples) and from the reference (R) site in Fjällbacka (ten samples). Analysis was performed on the top 200 genes in the ranked gene list according to FDR. The differential mRNA expression values are shown as log2 ratios with the color red indicating genes with a higher mRNA expression and green indicating genes with a lower mRNA expression in the harbor versus reference site.

supported by a principal component analysis, which separated the fish captured in Göteborg harbor from the fish captured at reference site (Fig. 2).

In the generated ranked gene list, genes coding for proteins known to be involved in the immune system had lower mRNA levels in the harbor compared to Fjällbacka. In particular genes involved in the innate immune defense, such as those coding for complement components and hepcidin (HAMP) (Table 2), had a lower differential expression. Furthermore, the mRNA expression levels of several genes involved in protein synthesis and folding were lower in fish collected from Göteborg harbor compared to fish

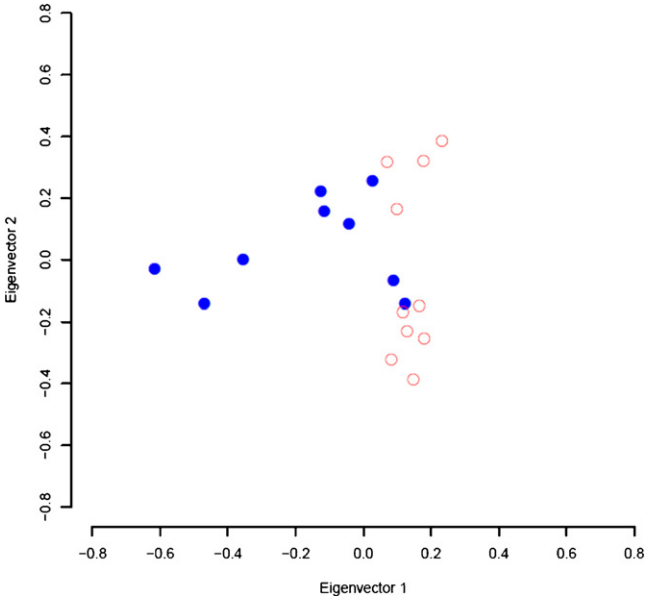


**Fig. 2.** Principal component analysis on hepatic transcriptome data from individual eelpout females captured in the polluted (●) waters of Göteborg harbor (nine samples) and from the reference (○) site in Fjällbacka (ten samples).

taken from the reference site. Among the induced genes in the harbor, we found several genes involved in apoptotic pathways such as diablo homolog and DNA-damage-inducible transcript 4 protein (DDIT4) as well as gene involved in detoxification (Table 2).

### 3.2. Gene Ontology term enrichment analysis

To identify affected pathways and differentially transcribed genes with similar biological functions, genes with differential mRNA expression were analyzed for the overrepresentation of GO terms (Table 3). When using the GO enrichment analysis and visualization tool (GOrilla) against the human database, several groups of biological process were found ($p < 0.001$), e.g., *protein folding* [GO:0006457], *humoral immune response* [GO:0006959] and *complement activation, classical pathway* [GO:0006958].

### 3.3. Verification of microarray data using quantitative PCR

To confirm the mRNA expression levels of genes identified via the microarray study, qPCR analyses were performed on RNA from the same individuals used for the microarray hybridization (Fig. 3). Nine genes were selected among the genes with most differential mRNA expression in the ranked gene list, and also from genes found in the GO enrichment analysis with a lower differential mRNA expression. The following nine genes were selected: cytochrome P450 24/1,25-dihydroxyvitamin D3-hydroxylase (CYP24), protein disulfide isomerase (PDI), signal recognition protein 9 kDa protein (SRP9), glucose-regulated protein, 78 kDa (HSPA5/BiP), DNA-damage-inducible transcript (DDIT4), diablo homolog (Diablo), hepcidin (HAMP), complement component 7 (C7) and lysozyme C (LysC). The qPCR analysis verified the mRNA levels measured from the microarray data (Fig. 3).

### 3.4. A follow up study conducted four years later on selected genes using quantitative PCR

To verify that the mRNA expression identified in the fish sampled 2006 reflected stable differences between the two sites, the mRNA expression of the nine selected genes was investigated by qPCR in fish caught four years later (Fig. 3). Five of these genes (SRP9, DDIT4, Diablo, HAMP and C7), showed similar differential mRNA expression in 2006 and in 2010 all with $p$-values < 0.05. PDI, HSPA5 and LysC had a lower differential mRNA expression in 2010 than in 2006; however, this differential expression was not significant ($p > 0.05$). Finally, the CYP24A gene with higher mRNA levels in eelpout females from Göteborg harbor captured in 2006 as compared to Fjällbacka, had lower mRNA levels in 2010, however not significantly ($p > 0.05$).

**Table 2**
Selected genes with differential mRNA expression in eelpout females captured from Göteborg harbor vs. eelpout females captured a the reference site in Fjällbacka.

| Accession no. | Description | Log 2 DE | *p*-Value | FDR |
|---|---|---|---|---|
| *DNA damage and apoptosis* | | | | |
| ZOVI0007782 | DNA-damage-inducible transcript 4 protein | 2.92 | 8.65E−08 | 2.56E−04 |
| ZOVI0033152 | Diablo homolog, mitochondrial precursor | 2.57 | 1.57E−06 | 1.99E−03 |
| ZOVI0022964 | Growth arrest and DNA-damage-inducible protein GADD45 beta | 2.25 | 1.78E−08 | 1.39E−04 |
| ZOVI0034162 | Proto-oncogene protein c-fos | 1.93 | 1.36E−06 | 1.99E−03 |
| ZOVI0043527 | Diablo homolog, mitochondrial | 1.75 | 1.88E−04 | 2.42E−02 |
| ZOVI0028606 | Growth arrest and DNA-damage-inducible protein GADD45 gamma | 1.11 | 5.86E−05 | 1.48E−02 |
| ZOVI0003438 | Serine/threonine-protein kinase Sgk1 | 1.41 | 5.40E−05 | 1.48E−02 |
| *Cellular respiration* | | | | |
| ZOVI0028293 | ATP synthase subunit b, mitochondrial | 1.96 | 4.27E−05 | 1.41E−02 |
| ZOVI0047802 | ATP synthase subunit epsilon, mitochondrial | 1.55 | 4.65E−04 | 3.36E−02 |
| ZOVI0001016 | Cytochrome c oxidase subunit 5B, mitochondrial | 1.19 | 1.55E−03 | 5.31E−02 |
| ZOVI0051334 | ATP synthase subunit g, mitochondrial | 1.12 | 1.59E−02 | 1.27E−01 |
| ZOVI0053333 | NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 2, mitochondrial | −0.97 | 2.33E−04 | 2.58E−02 |
| ZOVI0012472 | Cytochrome c oxidase polypeptide 7A2, mitochondrial | 1.06 | 9.12E−03 | 1.00E−01 |
| ZOVI0011258 | NADH-ubiquinone oxidoreductase chain 6 | −1.03 | 7.86E−03 | 9.53E−02 |
| *Positive regulation of cell growth* | | | | |
| ZOVI0045147 | Insulin-like growth factor-binding protein 1 | 1.55 | 3.24E−04 | 2.92E−02 |
| ZOVI0050931 | Insulin-like growth factor-binding protein 3 | 1.54 | 3.21E−04 | 2.92E−02 |
| ZOVI0017168 | Insulin-like growth factor I, adult form | −1.00 | 1.08E−02 | 1.07E−01 |
| *Vitamin D metabolic process* | | | | |
| ZOVI0038000 | CYP24A1 1,25-dihydroxyvitamin D(3) 24-hydroxylase, mitochondrial | 2.69 | 3.34E−04 | 2.94E−02 |
| ZOVI0006887 | 1,25-Dihydroxyvitamin D(3) 24-hydroxylase, mitochondrial Precursor | 2.07 | 1.10E−03 | 4.59E−02 |
| *Detoxification* | | | | |
| ZOVI0038124 | Liver carboxylesterase 22 | 2.59 | 7.44E−09 | 1.16E−04 |
| ZOVI0038123 | Carboxylesterase 7 | 2.57 | 2.52E−06 | 2.81E−03 |
| ZOVI0032461 | Cytochrome P450 3A30 | −1.77 | 7.96E−05 | 1.68E−02 |
| ZOVI0018586 | Cytochrome P450 3A13 | −1.05 | 2.59E−04 | 2.64E−02 |
| ZOVI0011605 | Cytochrome P450 2J5 | 1.04 | 4.14E−02 | 1.97E−01 |
| *Immune response* | | | | |
| ZOVI0046979 | Lysozyme C | −3.81 | 7.84E−06 | 6.45E−03 |
| ZOVI0005671 | Complement component C7 | −2.17 | 1.27E−06 | 1.99E−03 |
| ZOVI0028867 | Serum amyloid A-5 protein | −1.58 | 1.11E−03 | 4.59E−02 |
| ZOVI0038262 | Complement component 1 Q subcomponent-binding protein, mitochondrial | −1.48 | 2.17E−03 | 5.94E−02 |
| ZOVI0027991 | Collectin-12 | −1.43 | 6.13E−04 | 3.71E−02 |
| ZOVI0012537 | Serum amyloid A-3 protein | −1.42 | 1.11E−03 | 4.59E−02 |
| ZOVI0033472 | Complement C5 | −1.33 | 1.30E−03 | 4.94E−02 |
| ZOVI0029187 | Complement factor B | −1.23 | 4.26E−03 | 7.62E−02 |
| ZOVI0000384 | Complement C4 (fragments) | −1.12 | 3.98E−02 | 1.92E−01 |
| ZOVI0017427 | C4b-binding protein alpha chain | −1.11 | 2.96E−05 | 1.15E−02 |
| ZOVI0004499 | Complement component C8 beta chain | −1.01 | 1.09E−03 | 4.59E−02 |
| *Iron homeostasis* | | | | |
| ZOVI0038001 | Hepcidin | −3.19 | 6.63E−08 | 2.56E−04 |
| ZOVI0013021 | Haptoglobin | −1.36 | 8.77E−03 | 9.91E−02 |
| ZOVI0046516 | Hephaestin | −1.13 | 9.21E−03 | 1.01E−01 |
| ZOVI0053333 | Transferrin | −1.10 | 1.96E−02 | 1.40E−01 |
| *Protein transport and ER stress* | | | | |
| ZOVI0040061 | Heat shock 70 kDa protein 1 | −2.40 | 1.54E−06 | 1.99E−03 |
| ZOVI0049868 | Heat shock 70 kDa protein | −2.09 | 2.80E−05 | 1.14E−02 |
| ZOVI0038668 | 78 kDa glucose-regulated protein | −1.99 | 9.43E−04 | 4.36E−02 |
| ZOVI0050282 | Dolichol-phosphate mannosyltransferase | −1.92 | 2.47E−05 | 1.14E−02 |
| ZOVI0013079 | Signal peptidase complex catalytic subunit SEC11A | −1.67 | 4.87E−04 | 3.38E−02 |
| ZOVI0018899 | Stress-70 protein, mitochondrial | −1.53 | 4.30E−05 | 1.41E−02 |
| ZOVI0000208 | Protein disulfide-isomerase A4 | −1.54 | 2.21E−04 | 2.56E−02 |
| ZOVI0011442 | Protein disulfide-isomerase A6 | −1.53 | 3.89E−04 | 3.09E−02 |
| ZOVI0003888 | Calreticulin | −1.63 | 8.08E−05 | 1.68E−02 |
| ZOVI0022140 | UDP-N-acetylglucosamine–dolichyl-phosphate N-acetylglucosaminephosphotransferase | −1.52 | 1.29E−05 | 9.21E−03 |
| ZOVI0016973 | Endoplasmin | −1.42 | 3.14E−03 | 6.93E−02 |
| ZOVI0036724 | Signal recognition particle 9 kDa protein | −1.37 | 7.32E−03 | 9.20E−02 |
| ZOVI0036672 | Hypoxia up-regulated protein 1 | −1.24 | 1.55E−04 | 2.27E−02 |
| ZOVI0052852 | Signal recognition particle receptor subunit alpha | −1.22 | 5.59E−05 | 1.48E−02 |
| ZOVI0025258 | Protein disulfide-isomerase | −1.17 | 2.72E−03 | 6.54E−02 |
| ZOVI0009879 | Stress-associated endoplasmic reticulum protein 1 | −1.15 | 3.24E−02 | 1.76E−01 |
| ZOVI0048909 | ER lumen protein retaining receptor 3 | −1.02 | 1.76E−03 | 5.53E−02 |
| ZOVI0036375 | Signal recognition particle 19 kDa protein | −1.01 | 2.57E−04 | 2.64E−02 |
| *Protein synthesis* | | | | |
| ZOVI0013365 | Elongation factor G, mitochondrial | −1.52 | 1.19E−04 | 2.00E−02 |
| ZOVI0040437 | Eukaryotic translation initiation factor 4E-binding protein 1 | −1.35 | 3.77E−04 | 3.06E−02 |
| ZOVI0022783 | 60S ribosomal protein L7 | −1.33 | 1.22E−03 | 4.74E−02 |
| ZOVI0042117 | Eukaryotic translation initiation factor 4E-1A | −1.23 | 1.57E−02 | 1.26E−01 |
| ZOVI0000700 | Eukaryotic translation initiation factor 5 | −1.15 | 7.65E−04 | 4.00E−02 |

Table 2 (*Continued*)

| Accession no. | Description | Log 2 DE | *p*-Value | FDR |
|---|---|---|---|---|
| ZOVI0025302 | Eukaryotic translation initiation factor 6 | −1.13 | 1.65E−03 | 5.45E−02 |
| ZOVI0051985 | Eukaryotic peptide chain release factor subunit 1 | −1.10 | 2.82E−03 | 6.63E−02 |
| ZOVI0052421 | Eukaryotic translation initiation factor 3 subunit C | −1.08 | 9.14E−05 | 1.77E−02 |
| ZOVI0038186 | Probable ribosome biogenesis protein RLP24 | −1.06 | 1.44E−02 | 1.22E−01 |
| ZOVI0014147 | 60S ribosomal export protein NMD3 | −1.01 | 1.54E−03 | 5.30E−02 |
| ZOVI0020288 | Eukaryotic translation initiation factor 3 subunit B | −1.01 | 8.19E−03 | 9.59E−02 |
| *Aminoacyl-tRNA biosynthesis* | | | | |
| ZOVI0041372 | Purine nucleoside phosphorylase | −2.29 | 1.30E−05 | 9.21E−03 |
| ZOVI0041758 | Glycyl-tRNA synthetase | −1.77 | 2.61E−03 | 6.37E−02 |
| ZOVI0009441 | Tyrosyl-tRNA synthetase, cytoplasmic | −1.31 | 1.62E−04 | 2.31E−02 |
| ZOVI0016301 | Phenylalanyl-tRNA synthetase alpha chain | −1.28 | 6.13E−03 | 8.74E−02 |
| ZOVI0030220 | Aspartyl-tRNA synthetase, cytoplasmic | −1.13 | 4.74E−04 | 3.36E−02 |
| ZOVI0021639 | Glutaminyl-peptide cyclotransferase | −1.09 | 2.09E−05 | 1.14E−02 |
| ZOVI0048874 | Phenylalanyl-tRNA synthetase beta chain | −1.02 | 2.16E−03 | 5.94E−02 |

Selected genes with differential hepatic mRNA expression in eelpout captured in Göteborg harbor compared to fish captured at the reference site. The differential expression (DE) is presented as log2 DE. The false discovery rate (FDR) represents the portion of false positives.

### 3.5. Estimation of apoptotic hepatocytes in eelpout liver

To verify the differential mRNA expression of genes involved in apoptosis, the number of apoptotic hepatocytes in liver sections was estimated by using the TUNEL assay which fluorescently labels fragmented DNA in apoptotic cells. There was, however, no significant difference (*p*-value of 0.69) between the numbers of apoptotic cells in eelpout captured in the harbor ($1.22 \pm 0.19$ apoptotic cells per 1000 cells) or the reference site in Fjällbacka ($1.08 \pm 0.28$ apoptotic cells per 1000 cells).

## 4. Discussion

In this study, we used an oligonucleotide microarray to assess differential mRNA expression in eelpout collected from both a polluted and a reference site. Several genes involved in response to stress (apoptosis and DNA damage genes, immune responses genes, protein synthesis genes and drug metabolism and detoxification genes) had differential mRNA expression in fish collected from the two sites. Although it is not yet possible to conclusively link these responses to specific causes or stressors, the identities of many of the differentially expressed genes and pathways suggest that the differences in their mRNA expression levels may be due to exposure to contaminants in the harbor. Below, we will discuss in more detail the possible causes and significances of these affected genes and pathways. Importantly, our findings were verified by qPCR analyses in the same fish as well as in other eelpout collected in an independent sampling four years later, indicating that several of the most prominent differences identified by the microarray were not only correctly identified but also consistently different between the two sites over time. We also tried to link some of the differential mRNA expressions to protein function/phenotype (apoptosis in liver cells, blood parameters and liver somatic index (LSI)).

### 4.1. Lower mRNA expression of genes involved in the innate immune system

The innate immune system is the first line of defense, protecting the host from invading pathogens. The complement system is part of the innate immune response and involves over more than 25 proteins, most of which are synthesized by hepatocytes. The complement system is believed to have an important role in

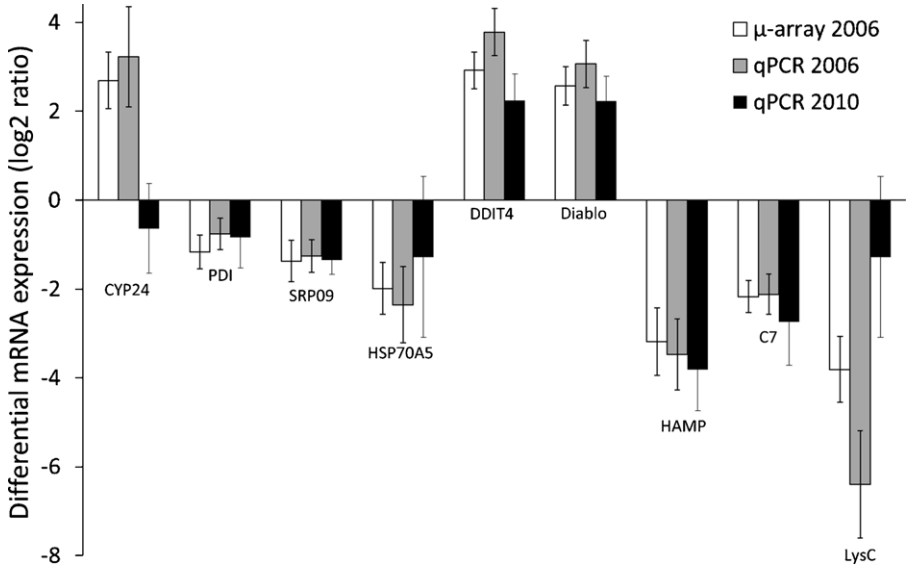


**Fig. 3.** Hepatic mRNA expression on selected genes in eelpout collected at a polluted site (Göteborg harbor) and a reference site (Fjällbacka). The mRNA expression was measured with microarray in fish sampled in 2006 (white bars) and quantitative real-time polymerase chain reaction analysis in fish sampled in 2006 and in 2010 (gray and black bars respectively). Values are expressed as log2 ratios of the differential mRNA expression in fish from Göteborg harbor compared with Fjällbacka. Error bars indicate ± SEM. The selected genes were: cytochrome P450 24/1,25-dihydroxyvitamin D3-hydroxylase (CYP24), protein disulfide isomerase (PDI), signal recognition protein 9 kDa protein (SRP9), glucose-regulated protein, 78 kDa (HSPA5/BIP), DNA-damage-inducible transcript (DDIT4), diablo homolog (Diablo), hepcidin (HAMP), complement component 7 (C7), and lysozyme C (LysC).

**Table 3**
Biological processes found in Gene Ontology (GO) term enrichment analysis.

| GO ID | Biological process | *p*-Value |
|---|---|---|
| A | | |
| GO:0000185 | Activation of MAPKKK activity | 2.89E−4 |
| GO:0051149 | Positive regulation of muscle cell differentiation | 3.87E−4 |
| GO:0016999 | Antibiotic metabolic process | 5.75E−4 |
| GO:0001649 | Osteoblast differentiation | 9.51E−4 |
| B | | |
| GO:0006987 | Activation of signaling protein activity involved in unfolded protein response | 2.54E−7 |
| GO:0030968 | Endoplasmic reticulum unfolded protein response | 1.00E−6 |
| GO:0006959 | Humoral immune response | 1.57E−4 |
| GO:0034374 | Low-density lipoprotein particle remodeling | 2.66E−4 |
| GO:0050715 | Positive regulation of cytokine secretion | 5.19E−4 |
| GO:0006457 | Protein folding | 6.29E−4 |
| GO:0006958 | Complement activation, classical pathway | 8.71E−4 |
| GO:0019348 | Dolichol metabolic process | 9.24E−4 |
| GO:0006418 | tRNA aminoacylation for protein translation | 9.64E−4 |

Gene Ontology (GO) term enrichment analysis revealed biological processes over-represented in the gene list with higher mRNA expression (A) and lower mRNA expression (B) in female eelpout captured in Göteborg harbor compared to the reference site in Fjällbacka. All differently expressed genes used for the enrichment analysis had a differential expression > 2 and FDR < 0.2.

the bacterial defense system of teleost fish (Holland and Lambris, 2002; Magnadóttir, 2006). Seven components of the complement system (C1q, C5, C4, C7, C8 beta chain, CFB and C4 binding protein) were differently expressed, and all were found to have decreased mRNA expression in eelpout from Göteborg harbor compared to those from the reference site. The most differentially expressed transcript identified in this study was the gene coding for lysozyme C (LysC), with mRNA levels 14 times lower in the harbor samples compared to the Fjällbacka samples. Lysozyme C is an enzyme with the ability to hydrolyze bacterial cell walls and is thus an important molecule in the innate immune system (Saurabh and Sahoo, 2008). The amount of lysozyme found in fish serum is an indicator of the status of the innate immune system (Watts et al., 2001). A low lysozyme activity has been suggested to be a valuable biomarker for environmental contaminants in integrating monitoring programs of flounder (*Platichthys flesus*) (Skouras et al., 2003a) and dab (*Limanda limanda L.*) (Skouras et al., 2003b) in the North Sea. Skouras et al. demonstrated that the lysozyme activity is inhibited in both flounder and dab collected from areas with high concentrations of contaminants such as PAH and DDT. These findings are consistent with the observed lower mRNA expression of the gene coding for lysozyme in eelpout captured at Göteborg harbor compared to fish captured at the reference site suggesting a lower immune response in eelpout in our study. The lower mRNA expression of lysozyme C in the harbor fish was however not confirmed when performing qPCR on eelpout sampled four years later (Fig. 3).

Several genes involved in iron homeostasis were differentially expressed in the harbor compared to Fjällbacka, including transferrin (TF), haptoglobin (HP) and hepcidin (HAMP). Transferrin is the main iron transporter protein found in blood plasma. A decrease in transferrin plasma levels can cause an iron overload as iron homeostasis is affected (Macedo and de Sousa, 2008). Haptoglobin is mainly synthesized by the liver and is released into the plasma to bind free hemoglobin, preventing the loss of iron via the kidneys (Wassell, 2000). Decreasing the levels of free haptoglobin in the blood could indicate liver damage or hemolytic anemia. Hepcidin is a hepatic protein with antibacterial activity, and its expression is induced following a bacterial challenge (Shike et al., 2002). Hepcidin is also expressed during iron overload (Hu et al., 2007) and binds to the iron export channel, called ferroportin causing degradation of the channel thus leading to increased iron storage. Transferrin, hepcidin and haptoglobin are believed to suppress bacterial growth by limiting the availability of free iron. The up-regulated mRNA expression of genes coding for iron regulatory proteins following bacterial infection in turbot and channel catfish has been previously reported in transcriptome analysis studies (Millan et al., 2011; Peatman et al., 2007). In our study, however transferrin, hepcidin and haptoglobin all were found to have lower mRNA expression levels in fish captured in the harbor compared to Fjällbacka which could indicate a higher susceptibility to bacterial infection in the harbor fish. The lower mRNA expression level of hepcidin observed in eelpout captured in Göteborg harbor was also verified by qPCR four years later (Fig. 3). In parallel monitoring studies on the very same individual eelpout used in this study, hematocrit (Ht) and hemoglobin (Hb) were analyzed (Förlin, 2008). Ht and Hb levels were high in the harbor compared to the reference site however, the ratios Hb/Ht were 23% (*p*-value of 0.046) or 31% (*p*-value of 0.0014) lower in the eelpout captured in the harbor compared to the reference site during 2006 and 2010, respectively (Förlin, 2008 and unpublished data) which could indicate a different regulation of hematopoiesis. Overall, this might be a consequence of a difference in oxygenation of blood among the eelpout captured at the different sites and could, in addition to the differential mRNA expression of transferrin, haptoglobin and hepcidin, indicated a difference in iron homeostasis.

Collectin-12 (COLEC12) is a member of the C-lectin family. In mammals, collectin-12 is associated with the defense against microorganisms and the protein facilitates the recognition and removal of pathogens by binding to carbohydrate structures on the invading microorganisms (Hansen and Holmskov, 1998). The mRNA levels of collectin-12 were lower in samples from Göteborg harbor compared to Fjällbacka. The function of collectin-12 in teleost fish is not known; however, if its function in fish is similar to its function in mammals, the lower collectin-12 mRNA expression would further indicate a higher susceptibility to bacterial invasion of the fish collected in Göteborg harbor.

The immunotoxicological effects of environmental contaminants, such as PAHs in fish are well known (Cuesta et al., 2011; Reynaud and Deschaux, 2006) and high concentrations of PAHs in bile from eelpout captured in Göteborg harbor has been reported earlier (Förlin, 2008; Sturve et al., 2005). Exposure to immunotoxic contaminants can lead to both suppression and activation of immune cells and proteins, and responses depend on type and concentration of the pollutant as well as on the species studied. Leaver et al. observed increased mRNA expression of genes involved in the innate immune system in European flounder chronically exposed to polluted estuarine sediments for seven months (Leaver et al., 2010). Falciani et al. found both higher and lower mRNA expression of genes involved in the immune system when performing large-scale genome-wide transcriptome studies on European flounder captured at five North Sea sampling sites with differing levels of contamination as compared to a more pristine reference site (Falciani et al., 2008). The indications of a lower activity in the innate immune system in eelpout collected in Göteborg harbor and its possible impact on health and performance of the fish living in this chronically polluted area need to be addressed in future studies. Our findings indicate the value of including biomarkers assessing the immunological health status of fish in monitoring studies.

### *4.2. Higher mRNA expression of genes involved in growth arrest and DNA damage*

Genes known to be involved in apoptosis, growth arrest and/or DNA damage were differentially transcribed between the sites (Table 2). Diablo homolog/SMAC was one protein with higher transcription levels in the harbor samples. Diablo homolog is a

mitochondrial protein released into the cytosol upon the initiation of apoptosis. This protein regulates apoptosis by activating caspases in responses to damage (Vucic et al., 2002). The diablo homolog has recently been postulated to be a novel biomarker of pollutant exposure (Leaver et al., 2010; Zacchino et al., 2012). Zacchino et al. showed that hepatic transcription levels of the diablo homolog were increased in European flounder assessed during an exposure study (using PAH and PCB) and higher in fish collected from contaminated estuaries compared to a reference site, indicating that the diablo homolog is a marker for both acute and chronic exposure. The DNA-damage-inducible transcript 4 (DDIT4) as well as the growth arrest and DNA-damage-inducible, beta and gamma (GADD45B and GADD45G, respectively) were all more highly transcribed in eelpout collected from the harbor (Table 3). These genes are known to be up-regulated in stressful growth arrest situations, and all are involved in regulation of growth and apoptosis (Fornace et al., 1992; Shoshani et al., 2002) and postulated to have both pro- and anti-apoptotic activities. The balance between pro- and anti-apoptotic proteins in a cell determines if a cell will undergo ether apoptosis or survive. The differential transcription of genes coding for proteins involved in both inhibition and activation of apoptosis in this study most likely reflect the different environmental conditions in these sites, including the mixture of toxic substances in the harbor. In the present study, the differential mRNA expression of the diablo homolog and DDIT4 was confirmed using qPCR for both the 2006 and the 2010 samples.

To link the differential mRNA expression of genes coding for proteins involved in apoptosis to phenotype, the number of TUNEL-positive apoptotic hepatocytes were studied in eelpout captured in the harbor and the reference site. As no significant differences in apoptotic cells were found, we cannot conclude that the differential mRNA expression of genes involved in the regulation of apoptosis reflects a higher prevalence of apoptosis in the liver of eelpout from the harbor. The assessment of apoptosis and DNA damage in eelpout has been previously used as an indicator of toxic exposure, as measured using different assays. Frenzilli et al. demonstrated increased levels of DNA strand-breaks (using the comet assay) and apoptotic erythrocytes (using the diffusion assay) in eelpout that were captured three weeks after an accidental bunker oil spill in Göteborg harbor in 2003 (Frenzilli et al., 2004). The application of apoptosis and DNA damage biomarkers was also demonstrated for eelpout sampled from the industrialized Tyne estuary (UK), which is known to be contaminated with high levels of PAHs. Lyons et al. (2004) demonstrated that germ cell apoptosis, indicated as TUNEL-positive cells and hepatic DNA adducts were increased in the Tyne compared to the less contaminated reference site at Alde. Schiedek et al. (2006) demonstrate that there were elevated number of DNA adducts in eelpout collected from the Wismar Bay area in the Baltic Sea, an area with high anthropogenic influences.

### 4.3. Differential mRNA expression of genes involved in protein synthesis and energy metabolism

Many genes coding for proteins involved in protein synthesis, such as translation (e.g., translation initiation factors), transport (e.g., signal recognition particles) and folding (e.g., disulfide-isomerases and 78 kDa glucose-regulated protein (HSPA5)) had lower mRNA levels in eelpout collected from Göteborg harbor compared to those collected in Fjällbacka (Table 2). This lower differential expression also reflects the lower differential mRNA expression of genes coding for several of the important plasma proteins known to be synthesized by the liver (e.g., complement components, transferrin, haptoglobin and insulin-like growth factor) (Table 2) in the eelpout collected in the harbor. Genes coding for proteins involved in ATP synthesis also have a differential mRNA expression in the sampled eelpouts, with both higher and lower mRNA expression of genes involved in cellular respiration (Table 2). These differential expressions could be a response to the chronic stress of living in a polluted habitat and might indicate an overall lower protein production in the liver and a differential energy metabolism in the harbor fish compared to those from the pristine reference site. A lower liver somatic index (LSI) has been noted in the harbor eelpout (LSI is 0.84 times lower in the harbor ($p = 0.02$) (Förlin, 2008) which might reflect a general lower activity in the liver in eelpout from Göteborg harbor. The lower mRNA expression levels of genes involved in protein synthesis were verified four years later by qPCR, using the signal recognition particle 9 kDa protein, however, not significant for glucose-regulated protein, 78 kDa or protein disulfide isomerase (Fig. 3). The differential mRNA expression in genes involved in protein folding are also known to be associated with stress response in the endoplasmic reticulum (the so-called ER stress response), which was also implicated by GO enrichment analysis when the response to unfolded proteins was observed. Proteins involved in the unfolded protein responses are known to be induced in the European flounder after acute exposure to toxins such as cadmium, PAH and PCB (Williams et al., 2006, 2007). However, the mRNA expression levels of proteins involved in folding were lower in eelpout from Göteborg harbor than from the reference site, indicating that the eelpout in the harbor are not responding in an acute manner to toxicants and may have instead adapted to a chronically polluted environment.

### 4.4. Differential mRNA expression of genes involved in drug metabolism and detoxification

During the parallel biomonitoring studies on eelpout captured in Göteborg harbor and Fjällbacka in November 2006, the hepatic detoxification enzyme activities of ethoxyresorufin-*O*-deethylase (EROD), glutathione reductase (GR), glutathione S-transferase (GST) and catalase were analyzed (Förlin, 2008). The only enzyme with a significantly different activity level between the sites was EROD, which was four times higher at the harbor ($p = 0.01$). EROD activity is known to be induced by compounds such as PAHs and dioxins, both of which are found in Göteborg harbor (Magnusson et al., 1996). In our transcriptome studies, the cytochrome P450 1A (CYP1A) gene (encoding for the enzyme primarily responsible for EROD activity) showed no significant differential mRNA expression between the two sites. The hepatic biomarkers (GR, GST and catalase) showed no elevated enzyme activity in the harbor compared to the reference site and no significant differential mRNA expression was observed for the respective genes in the microarray analysis.

Carboxylesterases constitute a large family of enzymes involved in drug metabolism and operate through the hydrolysis or transesterification of toxic compounds (Satoh and Hosokawa, 2006). In the present study, liver carboxylesterases EST22 and EST7 were found to have approximately six times higher mRNA expression levels in eelpout collected from Göteborg harbor compared to those collected in Fjällbacka, indicating a higher activation level of liver detoxification in eelpout captured in the PAH polluted harbor.

Other genes of the cytochrome P450 superfamily were also found to be differentially transcribed on the eelpout microarray. Five members of the CYP2 family had significantly higher mRNA expression levels in the harbor samples (with a differential expression between 1.6 and 2.1) and three subfamilies of the CYP3A family all had a significantly lower mRNA expression level (with a differential expression between 1.7 and 3.4) in the harbor samples. Both the CYP2 and CYP3 enzymes are involved in the metabolism of a large number of chemically diverse compounds including steroids, drugs and other xenobiotics (Schlenk et al., 2008). The regulation of these enzymes may well be the delicate result of both biotic and abiotic factors and may be influenced by exposure differences

within the complex mixture of compounds found in Göteborg harbor compared to Fjällbacka.

The CYP24A1 gene, also known as 1,25-dihydroxyvitamin D3-hydroxylase had a significantly higher mRNA expression level (by more than 6-fold) in eelpout from the harbor compared to those from the reference site. CYP24A1 is involved in regulating vitamin D levels by degrading the active form of vitamin D3 and is also known to play a role in calcium homeostasis (Sakaki et al., 2005). The higher CYP24A1 mRNA expression levels observed in this study were confirmed by qPCR. This higher mRNA expression was, however, not confirmed when performing qPCR on eelpout form both Göteborg harbor and Fjällbacka four years later. In a biomonitoring study on Atlantic female cod (*Gadus morhua*) captured in Stora Lungegårdsvann (Bergen, Norway), CYP24A1 mRNA expression levels were lower (Lie et al., 2009) as compared to cod captured at the reference site. Stora Lungegårdsvann, as Göteborg harbor, is an area with a high anthropogenic impact. The discrepancy in CYP24A1 mRNA expression between Göteborg harbor and Stora Lungegårdsvann suggests that the viability of CYP24A1 mRNA levels as a biomarker for use in field-sampled eelpout will require further evaluation in future studies.

## 5. Conclusions

Global transcriptome analysis can identify responses that are otherwise difficult to predict using traditional biomonitoring, where normally only a limited number of predetermined biomarkers are used. Large-scale genome-wide transcriptome profiling is thus an important tool in the search for new potential biomarkers. In this study, a large hepatic transcriptomic profiling on eelpout captured in Göteborg harbor and a reference site demonstrated the differential mRNA expression of genes and pathways involved in the innate immune system, drug metabolism, protein synthesis and apoptosis/DNA damage. These transcriptome analyses suggested that the eelpout in Göteborg harbor might have a lower ability to cope with bacterial infection and a lower production of important plasma proteins. The data also proposed that liver cells in eelpout from the harbor might be more prone to have accumulation of DNA damage and have differential expression of proteins involved in detoxification as a result of the environmental conditions in the harbor, including pollutants. The higher mRNA expression of genes involved in apoptosis was, however, not reflected in a higher prevalence of apoptotic cells in the livers of eelpout captured in the harbor area which emphasizes the importance of verifying transcriptomic data by linking suggested effects to protein function or phenotype. Some of the genes identified show promise as new biomarkers for use in future biomonitoring studies, as supported by consistent, site-specific expression differences in fish sampled four years later. However, future studies to validate the use of the potential biomarkers need to be performed as well as studies to verify the link between the molecular responses and the chronic exposure of the pollutants found in Göteborg harbor.

## Author contributions

NA planned the study, carried out the molecular biology including the microarray assays and drafted the manuscript. EK planned the study and performed all computational and statistical analyses on the microarray data. EA carried out the qPCR including the statistical analysis. DGJL and LF planned and supervised the study. All authors read, commented on and approved the final manuscript.

## Acknowledgments

This work was funded by the Swedish Research Council FORMAS, the Science Faculty strategic research platform "Ecotoxicology – from gene to ocean" (University of Gothenburg), the EU/BONUS funded BALCOFISH project, Swedish Environmental Protection Agency, the Swedish Research Council (medicine and health) and the Adlerbertska Research Foundation. We would also like to acknowledge Jari Parkkonen and Inger Holmqvist for technical assistance as well as Lina Gunnarsson and Filip Cuklev for valuable input with the microarray analyses.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2012.12.017.

## References

Baker, M.E., Ruggeri, B., Sprague, L.J., Eckhardt-Ludka, C., Lapira, J., Wick, I., Soverchia, L., Ubaldi, M., Polzonetti-Magni, A.M., Vidal-Dorsch, D., Bay, S., Gully, J.R., Reyes, J.A., Kelley, K.M., Schlenk, D., Breen, E.C., Sasik, R., Hardiman, G., 2009. Analysis of endocrine disruption in Southern California coastal fish using an aquatic multispecies microarray. Environmental Health Perspectives 117, 223–230.

Brack, K., 2002. Organotin compounds in sediments from the Goeta aelv estuary. Water, Air, and Soil Pollution 135, 131–140.

Brack, K., Stevens, R., 2001. Historical pollution trends in a disturbed, estuarine sedimentary environment, SW Sweden. Environmental Geology 40, 1017–1029.

Carney Almroth, B., Sturve, J., Berglund, A., Förlin, L., 2005. Oxidative damage in eelpout (*Zoarces viviparus*), measured as protein carbonyls and TBARS, as biomarkers. Aquatic Toxicology 73, 171–180.

Cuesta, A., Meseguer, J., Esteban, M.A., 2011. Immunotoxicological effects of environmental contaminants in teleost fish reared for aquaculture. In: Stoytcheva, M. (Ed.), Pesticides in the Modern World – Risks and Benefits. InTech, pp. 241–255.

Cuklev, F., Kristiansson, E., Fick, J., Asker, N., Förlin, L., Larsson, D.G.J., 2011. Diclofenac in fish: blood plasma levels similar to human therapeutic levels affect global hepatic gene expression. Environmental Toxicology and Chemistry 30, 2126–2134.

Falciani, F., Diab, A.M., Sabine, V., Williams, T.D., Ortega, F., George, S.G., Chipman, J.K., 2008. Hepatic transcriptomic profiles of European flounder (*Platichthys flesus*) from field sites and computational approaches to predict site from stress gene responses following exposure to model toxicants. Aquatic Toxicology 90, 92–101.

Fent, K., Sumpter, J.P., 2011. Progress and promises in toxicogenomics in aquatic toxicology: is technical innovation driving scientific innovation? Aquatic Toxicology 105, 25–39.

Fisher, M.A., Oleksiak, M.F., 2007. Convergence and divergence in gene expression among natural populations exposed to pollution. BMC Genomics 8, 108.

Fornace Jr., A.J., Jackman, J., Hollander, M.C., Hoffman-Liebermann, B., Liebermann, D.A., 1992. Genotoxic-stress–response genes and growth-arrest genes *gadd*, MyD, and other genes induced by treatments eliciting growth arrest. Annals of the New York Academy of Sciences 663, 139–153.

Frenzilli, G., Scarcelli, V., Del Barga, I., Nigro, M., Förlin, L., Bolognesi, C., Sturve, J., 2004. DNA damage in eelpout (*Zoarces viviparus*) from Göteborg harbour. Mutation Research 552, 187–195.

Förlin, L., 2008. Miljögifters effekter hos tånglake längs Bohuskusten 2006.

Gercken, J., Förlin, L., Andersson, J., 2006. Developmental disorders in larvae of eelpout (*Zoarces viviparus*) from German and Swedish Baltic coastal waters. Marine Pollution Bulletin 53, 497–507.

Gercken, J., Sordyl, H., 2002. Intersex in feral marine and freshwater fish from northeastern Germany. Marine Environmental Research 54, 651–655.

Hansen, S., Holmskov, U., 1998. Structural aspects of collectins and receptors for collectins. Immunobiology 199, 165–189.

Hedman, J.E., Rudel, H., Gercken, J., Bergek, S., Strand, J., Quack, M., Appelberg, M., Förlin, L., Tuvikene, A., Bignert, A., 2011. Eelpout (*Zoarces viviparus*) in marine environmental monitoring. Marine Pollution Bulletin 62, 2015–2029.

Holland, M.C., Lambris, J.D., 2002. The complement system in teleosts. Fish and Shellfish Immunology 12, 399–420.

Hu, X., Camus, A.C., Aono, S., Morrison, E.E., Dennis, J., Nusbaum, K.E., Judd, R.L., Shi, J., 2007. Channel catfish hepcidin expression in infection and anemia. Comparative Immunology, Microbiology and Infectious Diseases 30, 55–69.

Jacobsson, A., Neuman, E., Thoresson, G., 1986. The viviparous blenny as an indicator of environmental-effects of harmful substances. Ambio 15, 236–238.

Kristiansson, E., Asker, N., Förlin, L., Larsson, D.G.J., 2009. Characterization of the *Zoarces viviparus* liver transcriptome using massively parallel pyrosequencing. BMC Genomics 10, 345.

Larsson, D.G.J., Förlin, L., 2002. Male-biased sex ratios of fish embryos near a pulp mill: temporary recovery after a short-term shutdown. Environmental Health Perspectives 110, 739–742.

Leaver, M.J., Diab, A., Boukouvala, E., Williams, T.D., Chipman, J.K., Moffat, C.F., Robinson, C.D., George, S.G., 2010. Hepatic gene expression in flounder chronically exposed to multiply polluted estuarine sediment: absence of classical exposure 'biomarker' signals and induction of inflammatory, innate immune and apoptotic pathways. Aquatic Toxicology 96, 234–245.

Lehtonen, K.K., Schiedek, D., Kohler, A., Lang, T., Vuorinen, P.J., Förlin, L., Barsiene, J., Pempkowiak, J., Gercken, J., 2006. The BEEP project in the Baltic Sea: overview of results and outline for a regional biological effects monitoring strategy. Marine Pollution Bulletin 53, 523–537.

Lie, K.K., Lanzen, A., Breilid, H., Olsvik, P.A., 2009. Gene expression profiling in Atlantic Cod (*Gadus morhua L.*) from two contaminated sites using a custom-made cDNA microarray. Environmental Toxicology and Chemistry 28, 1711–1721.

Lyons, B.P., Bignell, J., Stentiford, G.D., Feist, S.W., 2004. The viviparous blenny (*Zoarces viviparus*) as a bioindicator of contaminant exposure: application of biomarkers of apoptosis and DNA damage. Marine Environmental Research 58, 757–761.

Macedo, M.F., de Sousa, M., 2008. Transferrin and the transferrin receptor: of magic bullets and other concerns. Inflammation and Allergy Drug Targets 7, 41–52.

Magnadóttir, B., 2006. Innate immunity of fish (overview). Fish and Shellfish Immunology 20, 137–151.

Magnusson, K., Ekelund, R., Dave, G., Granmo, A., Förlin, L., Wennberg, L., Samuelsson, M., Berggren, M., Brorstrom-Lunden, E., 1996. Contamination and correlation with toxicity of sediment samples from the Skagerrak and Kattegat. Journal of Sea Research 35, 223–234.

Millan, A., Gomez-Tato, A., Pardo, B.G., Fernandez, C., Bouza, C., Vera, M., Alvarez-Dios, J.A., Cabaleiro, S., Lamas, J., Lemos, M.L., Martinez, P., 2011. Gene expression profiles of the spleen, liver, and head kidney in turbot (*Scophthalmus maximus*) along the infection process with *Aeromonas salmonicida* using an immune-enriched oligo-microarray. Marine Biotechnology 13, 1099–1114.

Moran, P.W., Aluru, N., Black, R.W., Vijayan, M.M., 2007. Tissue contaminants and associated transcriptional response in trout liver from high elevation lakes of Washington. Environmental Science and Technology 41, 6591–6597.

Oleksiak, M.F., 2008. Changes in gene expression due to chronic exposure to environmental pollutants. Aquatic Toxicology 90, 161–171.

Peatman, E., Baoprasertkul, P., Terhune, J., Xu, P., Nandi, S., Kucuktas, H., Li, P., Wang, S., Somridhivej, B., Dunham, R., Liu, Z., 2007. Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium. Developmental and Comparative Immunology 31, 1183–1196.

Reynaud, S., Deschaux, P., 2006. The effects of polycyclic aromatic hydrocarbons on the immune system of fish: a review. Aquatic Toxicology 77, 229–238.

Roling, J.A., Bain, L.J., Gardea-Torresdey, J., Key, P.B., Baldwin, W.S., 2007. Using mummichog (*Fundulus heteroclitus*) arrays to monitor the effectiveness of remediation at a superfund site in Charleston, South Carolina, USA. Environmental Toxicology and Chemistry 26, 1205–1213.

Ronisz, D., Larsson, D.G.J., Forlin, L., 1999. Seasonal variations in the activities of selected hepatic biotransformation and antioxidant enzymes in eelpout (*Zoarces viviparus*). Comparative Biochemistry and Physiology: Part C 124, 271–279.

Sakaki, T., Kagawa, N., Yamamoto, K., Inouye, K., 2005. Metabolism of vitamin D3 by cytochromes P450. Frontiers in Bioscience 10, 119–134.

Satoh, T., Hosokawa, M., 2006. Structure, function and regulation of carboxylesterases. Chemico-Biological Interactions 162, 195–211.

Saurabh, S., Sahoo, P.K., 2008. Lysozyme: an important defence molecule of fish innate immune system. Aquaculture Research 39, 223–239.

Schiedek, D., Broeg, K., Barsiene, J., Lehtonen, K.K., Gercken, J., Pfeifer, S., Vuontisjarvi, H., Vuorinen, P.J., Dedonyte, V., Koehler, A., Balk, L., Schneider, R., 2006. Biomarker responses as indication of contaminant effects in blue mussel (*Mytilus edulis*) and female eelpout (*Zoarces viviparus*) from the southwestern Baltic Sea. Marine Pollution Bulletin 53, 387–405.

Schlenk, D., Celander, M., Gallagher, E.P., George, S., James, M., Kullman, S.W., van den Hurk, P., Willett, K., 2008. Biotransformation in fishes. In: Di Giulio, R.T., Hinton, D.E. (Eds.), The Toxicology of Fishes. CRC Press, pp. 55–152.

Shike, H., Lauth, X., Westerman, M.E., Ostland, V.E., Carlberg, J.M., Van Olst, J.C., Shimizu, C., Bulet, P., Burns, J.C., 2002. Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge. European Journal of Biochemistry 269, 2232–2237.

Shoshani, T., Faerman, A., Mett, I., Zelin, E., Tenne, T., Gorodin, S., Moshel, Y., Elbaz, S., Budanov, A., Chajut, A., Kalinski, H., Kamer, I., Rozen, A., Mor, O., Keshet, E., Leshkowitz, D., Einat, P., Skaliter, R., Feinstein, E., 2002. Identification of a novel hypoxia-inducible factor 1-responsive gene, RTP801, involved in apoptosis. Molecular and Cellular Biology 22, 2283–2293.

Skouras, A., Broeg, K., Dizer, H., von Westernhagen, H., Hansen, P.D., Steinhagen, D., 2003a. The use of innate immune responses as biomarkers in a programme of integrated biological effects monitoring on flounder (*Platichthys flesus*) from the southern North Sea. Helgoland Marine Research 57, 190–198.

Skouras, A., Lang, T., Vobach, M., Danischewski, D., Wosniok, W., Scharsack, J.P., Steinhagen, D., 2003b. Assessment of some innate immune responses in dab (*Limanda limanda L.*) from the North Sea as part of an integrated biological effects monitoring. Helgoland Marine Research 57, 181–189.

Strand, J., 2007. Biologisk effektmonitering, kapitel 14, Marine områder 2005–2006. Faglig rapport fra DMU 639. Tilstand of udvikling i miljø-og naturkvaliteten. Danmarks Miljøundersøgelser. Aarhus universitet.

Strand, J., Andersen, L., Dahllöf, I., Korsgaard, B., 2004. Impaired larval development in broods of eelpout (*Zoarces viviparus*) in Danish coastal waters. Fish Physiology and Biochemistry 30, 37–46.

Strand, J., Bossi, R., Dahllöf, I., Jensen, C.A., Simonsen, V., Tairova, Z., Tomkiewicz, J., 2009. Dioxin og biologisk effektmonitoring i ålekvabbe i kystnære danske farvande. Danmarks Miljøundersøgelser. Aarhus universitet. DMU 743.

Sturve, J., Berglund, A., Balk, L., Broeg, K., Bohmert, B., Massey, S., Savva, D., Parkkonen, J., Stephensen, E., Koehler, A., Förlin, L., 2005. Effects of dredging in Goteborg harbor, Sweden, assessed by biomarkers in eelpout (*Zoarces viviparus*). Environmental Toxicology and Chemistry 24, 1951–1961.

Van Aggelen, G., Ankley, G.T., Baldwin, W.S., Bearden, D.W., Benson, W.H., Chipman, J.K., Collette, T.W., Craft, J.A., Denslow, N.D., Embry, M.R., Falciani, F., George, S.G., Helbing, C.C., Hoekstra, P.F., Iguchi, T., Kagami, Y., Katsiadaki, I., Kille, P., Liu, L., Lord, P.G., McIntyre, T., O'Neill, A., Osachoff, H., Perkins, E.J., Santos, E.M., Skirrow, R.C., Snape, J.R., Tyler, C.R., Versteeg, D., Viant, M.R., Volz, D.C., Williams, T.D., Yu, L., 2010. Integrating omic technologies into aquatic ecological risk assessment and environmental monitoring: hurdles, achievements, and future outlook. Environmental Health Perspectives 118, 1–5.

Wassell, J., 2000. Haptoglobin: function and polymorphism. Clinical Laboratory 46, 547–552.

Watts, M., Munday, B.L., Burke, C.M., 2001. Immune responses of teleost fish. Australian Veterinary Journal 79, 570–574.

Williams, T.D., Diab, A.M., George, S.G., Godfrey, R.E., Sabine, V., Conesa, A., Minchin, S.D., Watts, P.C., Chipman, J.K., 2006. Development of the GENIPOL European flounder (*Platichthys flesus*) microarray and determination of temporal transcriptional responses to cadmium at low dose. Environmental Science and Technology 40, 6479–6488.

Williams, T.D., Diab, A.M., George, S.G., Sabine, V., Chipman, J.K., 2007. Gene expression responses of European flounder (*Platichthys flesus*) to 17-beta estradiol. Toxicology Letters 168, 236–248.

Williams, T.D., Turan, N., Diab, A.M., Wu, H.F., Mackenzie, C., Bartie, K.L., Hrydziuszko, O., Lyons, B.P., Stentiford, G.D., Herbert, J.M., Abraham, J.K., Katsiadaki, I., Leaver, M.J., Taggart, J.B., George, S.G., Viant, M.R., Chipman, K.J., Falciani, F., 2011. Towards a system level understanding of non-model organisms sampled from the environment: a network biology approach. PLoS Computational Biology, 7.

Vucic, D., Deshayes, K., Ackerly, H., Pisabarro, M.T., Kadkhodayan, S., Fairbrother, W.J., Dixit, V.M., 2002. SMAC negatively regulates the anti-apoptotic activity of melanoma inhibitor of apoptosis (ML-IAP). The Journal of Biological Chemistry 277, 12275–12279.

Zacchino, V., Minghetti, M., Centoducati, G., Leaver, M.J., 2012. Diablo/SMAC: a novel biomarker of pollutant exposure in European flounder (*Platichthys flesus*). Ecotoxicology and Environmental Safety 79, 176–183.